METHOD AND DEVICE FOR DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATIONS AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority of PCT/N095/00052, filed Mar. 10, 1995, now WO 95/24648, and Norwegian application no. 940866, filed Mar. 10, 1994.

The present invention relates to an immunomagnetic method for detection of specific target cells in cell populations and solutions of cell populations. The invention also relates to a kit and apparatus for performing the method in different cell populations.

In biology, biochemistry and adjacent fields there is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and isolated and purified. Furthermore, the cell content may be examined using a range of sophisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sophisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen-antibody, enzyme-receptor, ligand-receptor interactions on cells and biotin-avidin binding, of which antigen-antibody binding is most frequently used.

When such methods are used for isolation of specific cells, which are the subject for farther various examinations it is necessary that the cells should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for providing physiological conditions such that the isolated specific cells can develop in sufficient numbers to allow further characterisation.

Methods are known in which one of the binding partners is attached to an insoluble support., such as paramagnetic particles, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the unwanted cells. Following the incubation the cell/antibody/particle complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells and are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (WO91/01368) and relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, antihapten antibodies and anti-cell antibodies to bind

to each other, thus constructing a linkage between the insoluble support. i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

Furthermore, WO91/09938 describes the use of a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haematopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles. WO92/04961 comprises a method and a complicated equipment to separate cells or different molecules from a non-magnetic test medium by using colloidal magnetic paricles. In this method small (sub micron) particles are used because it is necessary to avoid precipitation of the particles in the solution and this method necessitates complicated apparatus, in which magnetic intensifying means is immersed in the test medium. This may have adverse effects on the cells.

In "Application of Magnetic Beads in Bioassays", B. Haukanes and C. Kvam. Bio/Technology, 11:60–63. 1993,. several methods are described for use of magnetic particles to remove tumor cells from bone marrow, isolation of lymphoid cells from peripheral blood and isolation of DNA, RNA and DNA-binding proteins. All described methods have specificities which are unsuitable for the present purpose of detecting only target-cells. The above methods will in addition to target cells also bind non-target cells due to cross-reactivity and unspecific adhesion of the antibody-particle complex.

There is also described a multiwell filtration apparatus for the assay of microliter quantities (EP-A-0 098 534), a filter strip and composite assemblies for filtering microliter quantities of fluid (EP-A-0 339 769) and an assay cartridge which has a substantially rectangular base plate, a substantially rectangular top plate and four side walls (EP-A-0 131 934). None of the above apparatus are applicable for the present purpose in that they describe pore sizes which are too small for the present purpose of retaining only particle-cell rosettes. Furthermore the filters are not designed to be exposed to several examinations of the retained cells without removing them form the filter medium.

There is a need for a simple linkage to connect a target cell to an insoluble support, which does not involve compounds of a rather unspecified hapten-group, and which is directed to detection of specific target cells, with a mini-mum of unspecific cell association and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

In a co-pending application by one of the applicants (WO94/07 139, filed Sep. 10, 1993) a method is described for detecting diagnostic purposes specific target cells without the problem with unspecific binding to normal cells. They represent sensitive detection methods for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and simple. Furthermore, the methods can be used for isolation of cells for biochemical, biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex.

There is, however, a need for improvements such as isolation of the particle-bound target cells in the target cell suspension, from unbound beads, unspecifically bound non-target cells and unbound non-target cells, which is simple to perform, not time requirering and with render the target cell/particle complexes suitable to perform further analysis such as for example microscopic examinations and growing in a culture medium.

BRIEF SUMMARY OF THE INVENTION

These objects are obtained by the present invention outlined by the method, apparatus and kit characterised in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cell populations is suitable for detection, but may also be used in positive isolation of specific types of both normal cells and pathogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as paramagnetic particles, which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood. Furthermore, incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method.

Furthermore, according to the present invention, the method can be profoundly improved and simplified by transfering the supension of target cell/particle complexes to the cell filtering device or cell separator according the present invention and the total number of target cells viewed microscopically or grown in a physiologically base culture medium to be characterised for the presence of specific biochemical and biological features.

BRIEF DESCRIPTION OF THE DRAWINGS

Of the drawings:

FIG. 1.1. shows a perspective view of an embodiment of the cell filtering device or Cell Separator, partly assembled.

FIG. 1.2. shows a perspective view of another embodiment of the Cell Separator, partly assembled.

FIG. 2. shows a perspective view of a version of the Cell Separator Multiwells wit

FIG. 3. shows membrane Filter detached from the Multiwells.

FIG. 4. shows a perspective view of a version of the culture dish with lid arrangement for the Cell Separator Multiwells and/or the Membrane Filter.

FIG. 4*a*. shows a side elevation of the Multiwell arrangement in the culture dish.

FIG. 5. shows a perspective view of a version of the Cell Separator Fitrate Collection Box.

FIG. 6. show melanoma cell-particle rosettes entrapped on cell filter device using the method described.

DETAILED DESCRIPTION

In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and possible isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

In the management of cancer patients, the staging of the disease with regards to whether it is localized or if metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cyto-centrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and immunofluorescence. Thus, slides prepared from biopsied tumors or cyto-centrifugation have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized calorimetrically or by fluorescence. The latter method requires the use of a fluorescence microscope, alternatively preparing a cell suspension and use of a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flow cytometric examinations also involve expensive equipment.

The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence. Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1% of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a microscope, but requires the availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor cells, since a large volume and high number of cells can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The method and apparatus described provides a solid support and permenant record which is easily viewed by microscopy, permits assessment and quantification of the whole specimen rather than small fractions thereof and allows the use of large specimen volumes to be analysed, the device may also be scanned automatically by conventional densitometric technology. The monoclonal antibodies used bind with sufficient specificity to, for example, tumor cells and not to other cells than the target cells present in mixed cell suspensions, like blood, bone marrow, and in other

tumor manifestations, such that all cells with attached beads represent the target-cells. In addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on the normal cells in question, or for other purposes to specified subpopulations of normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC31), Cluster 2 (MW-40 kD) antigen (NrLulO) (Myklebust et al. Br. J. Cancer Suppl. 63, 49–53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27–36, 1981), 80 kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302–5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insulin receptor, insulin-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells, in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological pulmonary, autoimmune, gastrointestial, genitourinary, reticuloendothelial and other disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. A more complete list of the antigen determinants and the corresponding antibodies or antibody fragments used in the present improved method is presented in Table 1.

Methodology

The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibodycoated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5–10 min to 2 h, preferably for 30 min at 0–25C., preferably at 4C., under gentle rotation. The present method may also be performed in a changed order of steps, in that the free target-cell antibodies are added to the cell suspension, incubated for 5–10 min to 2h, preferably 30 min, at 0–20C., preferably 4C., under gentle rotation. The paramagnetic particles, precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5–10 min to 2 h, preferably 30 min, at 0–25C., preferably 4C. under gentle agitation. The present method may also be performed in an abbreviated number of steps, in that the free target-cell antibodies are added to the cell suspension, at the same time and together with the precoated paramagnetic particles and subjected to incubation of 5–10 min to 2 h, preferably 30 min, at 0–25C., preferably 4C. under gentle agitation. The number of antibody-coated beads added to the cell suspension should be between 0.5–10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1–10% of the total number of cells. For specific purposes, and in the cases where the density of the target-cells is low, for example malignant cells, or the target-cells represent a very low fraction of the total number of cells (about 1%), the target cells can be positively separated from non-target cells in a magnetic field. The isolated target cells, in cell suspension may then be transferred to a cell counting device, and the number of cells with attached beads may be determined by microscopy. The present method may also be performed, and preferably so, by transferring the isolated target cell suspension to the cell filtering device described in this application, and the total number of isolated target cells viewed by microscopy. The isolated target cells in the filter device may be fixed and stained to facilitate viewing by light microscopy. For specific purposes and in cases where the isolated target cells are required to be functionally active, a physiologically based culture medium may be added to the cell filter device and subjected to incubation for an unspecified time at 37C. The isolated target-cells may be grown and subsequently characterised for the presence of specific biochemical and biological features. Moreover, the target-cells may be characterised for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle-target cell linkage. For several purposes it is of interest to examine specific genes in a pure population of target cells at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissues, central nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity. With the methods of prior art, signals obtained on Southern, Northern and

Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the target-cells only. This also relates to for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerase chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.

The application of the new method steps may differ depending on type of tissues to be examined.

a) Tissue from solid or needle tumor biopsies is prepared mechanically or with mild enzymatic treatment into a single cell suspension, to which the primary, specific antibodies or antibody fragments are added directly or after washing the cell suspension with phosphate buffered saline or culture medium with or without serum, such as fetal calf serum, bovine, horse, pig, goat or human serum.

b) If the material is a sample of pleural or ascitic effusion, cerebrospinal fluid, urine, lymph or body fluids such as effusions in the joints of patients with various forms of arthritis, the specific antibodies or antibody fragments are either added to the samples directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and brought back into suspension,

c) If the material consists of blood or bone marrow aspirate, the specific antibodies or antibody fragments are either added to the samples directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and brought back into suspension, or a mononuclear cell fraction may be prepared by gradient centrifugation on e.g. Lymphoprep before washing, resuspension, and addition of the appropriate antibodies or antibody fragments.

The procedure conditions for a) and by are established, as exemplified by results obtained in successful experiments as those described below.

For c) the results have been found to be influenced by a high number of factors which have been examined in detail. Among these are antibody concentration, the ratio of the number of paramagnetic particles versus number of cells, incubation times and volumes, type of incubation medium, and the pH level. The particle to mononuclear cell ratio in all experiments should be in the range of 0.5/1–2/1, depending on the binding affinity of the primary specific antibodies or fragments.

A major problem has been unspecific attachment to normal blood or bone marrow cells of particles coated with either sheep or rat anti-mouse antibodies alone. or in addition with the specific antibodies. Experiments have shown that the unspecific binding is equally high without the presence of the specific antibodies, indicating that the problem is not caused by crossreactivity of the targeting antibodies to normal cells. The possibility that the less than optimal specificity could be caused by ionic binding has been ruled out. Another possibility was that subpopulations of normal cells of the B-lineage might adhere to the particle-antibody complexes. However, immunomagnetic removal of B-cells from the cell suspension before adding the specific antibodies/antibody-particle complexes did not improve the specificity of the latter.

The problem with the procedure used on isolated mononuclear fractions of bone marrow and peripheral blood, that some non-target cells might also bind paramagnetic particles, has been circumvented or overcome. Thus with sheep-anti-mouse antibody coated particles alone or with specific antibodies the number of particles unspecifically attached to a low fraction mononuclear blood or bone marrow cells was reduced from an average of 10 to about 1 and in parallel the fraction of normal cells with particles decreased from 1–2% to 0.5–1% or less.

Evidence has been obtained that the problem may be caused by hydrophobic forces associated with the antibodies bound to the paramagnetic particles. Methods for reducing this hydrophobicity is thus claimed. One such method is pre-incubation of the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, for example Tween 20™ in concentrations of less than 0.1% for 30 minutes at 4C. When possible selection of the target cells is warrented, the cell suspension should contain a low concentration of the detergent, e.g. 0.01% of TWEEN 20™ polyoxyethylenesorbitan monolaurate. In several experiments this procedure has almost eliminated or dramatically reduced the problem of unspecific binding seen with the mononuclear cell fractions from blood or bone marrow.

The other improvement which, if found warranted, may be used together with the detergent step as follows:

After incubation of the cell suspension with the primary antibodies or antibody fragments and the antibody-coated paramagnetic particles as described in previously, the cell suspension is incubated with a second set of antibodies or antibody fragments directed against other extracellular or against intracellular determinants of the target cells, with or without pre-treatment with cell fixatives such as formaldehyde or alcohols. These antibodies or their fragments should have been prelabeled by fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or enzymes like peroxidase and alkaline phosphatase, allowing visualization by per se known methods in the microscope and/or a suitable counting device.

The target cells will both be visualized with the latter method and have bound particles to their surface, and can thus be enumerated.

To simplify the distinction between non-target and target cells, the cell suspension, or part thereof, can before the second visualization step either be subjected to cytospin centrifugation or portions of the suspension are attached to coated glass slides on which the particle-bound cells will be spread out in a thin layer, facilitating the recognition of the double-"stained" cells.

An alternative method according to the present invention to further simplify the distinction between non-target and target cells comprises the cell filter device, wherein the whole cell suspension after the target cell selection steps, can be added directly to the cell filter device. The free unbound beads, unspecifically bound non-target cells, and any unbound non-target cells, will pass through the filter leaving the bound target cells to be visualized on the filter. The filter with the isolated target cells can be removed from the device and the cells may be fixed and stained using known immunohistochemical methods and viewed by microscopy. After the filter has been removed from the device it can be treated as a conventional microscope slide of the type that is known and normally used in immunohistochemistry.

For specific purposes the filter may either be removed from the device or remain integral to the device, and a culture medium added, such as any known culture medium with or without agarose, for the purpose of propagating the isolated target cells situated on the filter.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic particles pre-coated with IgG isotype specific anti-mouse or anti-human antibody as one part of it, and different target cell-associated, for example tumor cell, antibodies as another part. In a third embodiment the kit contains paramagnetic particles precoated with specific anti-Fc antibodies, such as polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-mouse, or anti-human antibodies, capable of binding to the Fc portion the target-cell associ-

ating antibodies, bound to specific anti-target-cell antibodies. In a fourth embodiment the kits contain distinctive particles of a paramagnetic or non-magnetic nature, which may be coated or uncoated with a target-cell antigen or group of target-cell antigens, such that when processed by the method these particles become entrapped in the cell filter device, thereby acting as a control in demonstrating for example that all aspects of the antibody-antigen interactions in the method are working correctly. These particles may be incorporated into the cell suspension at a stage before or during the method, or the particles may be used as a separate "cell suspension" to be processed using the same method as the cell suspension comprising the target cells to be separated. In a further embodiment the kit contains other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target-cells, where said antibodies or antibody fragments are conjugated to peroxidase, alkaline phosphatase, or other enzymes, together with relevant substrates to such enzymes, or where said antibody or antibody fragment is bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

Apparatus

The new feature of the method concerns a cell filter device, which may also be termed a multiwell cell separator, and may or may not be a part of the kit as described. The device concerns a microwell cell separator arrangement, which is used to separate mixed populations of different sized cells, such as those found in blood or bone marrow. The resulting cells can be viewed directly on the membrane by microscopy or automated scanning devices. This invention may be used in conjunction with conventional magnetic particle cell isolation techniques to provide a rapid, sensitive, and simple method for screening large numbers of high or low volume samples for the presence of tumour cells within 1 to 4 hours.

According to the present invention there is provided a microwell cell separator arrangement comprising an open topped filtrate collection box, which may or may not have an attachment for a vacuum tube, and has a removable and disposable multiple wells arrangement with a cell separating membrane filter which forms the base of these multiple wells. A lid or cover to this arrangement may also be provided for.

The filtrate collection box and lid arrangement may be made from a material suitable for high temperature sterilisation, or may be made from a plastic transparent or opaque plastic material such as is known for tissue culture plastic wares.

The cell separating membrane filter may comprise a regular and consistent pore shape and size, such as is found in nylon monofilament membranes, which forms the base of the individual wells. The cell separating membrane filter may be secured to the microwells such that it can be removed after the cell separation method in order to facilitate examination. The cell separating membrane may also comprise a card or plastic surrounding frame to facilitate examination after removal from the microwells.

The filtrate collection box may comprise a frame in which removable strips of more than one well may be inserted.

The filtrate collection box may be fashioned similar to a conventional 96-well plate adapted to accommodate the cell separating membrane, collection box and low pressure vacuum attachment.

The invention may also comprise an upper lid or cover.

A disposable culture dish with lid is provided for in the device that allows the microwell strips to be inserted and cultured asceptically. Integral to the culture dish are indentations or notches that facilitate the positioning of the microwell strip similar to that in the filtrate collection box, and to prevent movement of the microwell strip during culture. The indentations or notches as described may or may not also provide for the location of the cell separating membrane after removal from the microwell strip.

The invention will be further apparent from the following description with reference to the figures of the accompanying drawings, which show, by way of example only, one form of the microwell cell separator arrangement embodying the same.

Referring to FIGS. **1.1**, **1.2**, **2**, **3**, **4.1**, **5**, and **6** of the drawings it will be seen that the Microwell Cell Separator arrangement **20** consists of a lid or cover **21** and a filtrate collection box **22**, which may or may not have a low pressure vacuum attachment port **23**, with removable Multiwell strips **24** which have a detachable membrane base **25** with support **25***a*.

FIGS. **1.1**. and **1.2**. shows two alternative embodiments of the invention partially assembled.

The filtrate collection box **22** may be similar in some respects to conventional 96-well plate formats with removable well strips, and may be arranged to fit one or multiple strips of wells.

The Multiwells **24** may be arranged in double strips as shown or in single or multiple strips.

The engagement of the Multiwells **24** in the Filtrate Collection Box **22** is such that only one orientation is possible, which may be provided for by locating pins **28** or notches **29**.

The Cell Separator Membrane Filter **25** is fixed to the bottom of the Multiwells **24** and forms the base of the wells. The fixing of the membrane filter **25** to the Multiwells **24** is such that they can be separated without deformation of the membrane filter **25** or the membrane filter support **25***a*.

The membrane filter **25** can be viewed by microscopy or may be scanned by is densitometric or similar methodology.

The membrane filter **25** may comprise a regular and consistent pore shape and size, such as is found in nylon monofilament membranes, which forms the base of the individual wells, and may be of 5–75 μm pore size but preferably 20 μm.

The Multiwells **24** within or without the Filtrate Collection Box **22** may also be made of a material suitable for tissue culture purposes, which may also be suitable for viewing in conventional 96-well plate scanning or plate reading machines.

The culture dish **26** and culture dish lid **27** may also be made of a material suitable for tissue culture purposes. In this way it is possible to supply culture medium both through the top of the multiwells and in the bottom of the culture dish **26**.

All dimensions shown in the figures are exemplary and the cell filtering device **20** should not be limited by these dimensions. Furthermore, it will be appreciated that it is not intended to limit the invention to the above example only, many variations being possible without departing from the scope thereof. The present method will in the following be illustrated by model experiments, examples of the usefulness of the new method and examples of practical applications. These examples shall not be regarded as in any way limiting the invention.

Model Experiments

1. Binding of antibody-bead complexes to tumor cell lines. To determine antibody 5 concentrations and optimal conditions for the binding of antibody-paramagnetic particle complexes to tumor cells, a large panel of cancer cell lines was used. The paramagnetic beads were bound to the cells, either by coating the specific antibodies to sheep-anti-mouse antibody (SAM)-coated paramagnetic particles, or by first incubating the cells with the specific antibodies, washing, followed by a second incubation with SAM-coated particles. The results of these experiments are given in Tables 2a and 2b, in which + indicates binding of several beads to all cells, (+) indicates either a lower number of beads bound to each cell, or that not all the tumor cells had beads attached to their surface, whereas reflects no binding, and (–) indicates very weak binding.
2. Detection of tumor cells in the mononuclear fraction of bone marrow or peripheral blood. Model experiments were performed where specific antibodies and SAM-coated paramagnetic particles were added either to such mononuclear cells or to a cell suspension where a different number of cancer cells from in vitro cultivated cell lines were added to said mononuclear cells. In some experiments, either the mononuclear cells, or the malignant cells were prestained with a fluorescent dye, to be able to distinguish between the two types of cells. In all experiments, non-binding primary antibodies, and/or sheep-anti-mouse antibody-coated beads were used separately as controls. Additional experiments without the preparation of a mononuclear cell fraction of peripheral blood were performed. It was found that the separation of cells in this way reduced the amount of unspecific binding compared to the Lymphoprep separated blood fractions.
3. Separation and visualisation of antibody-bead complexes to tumor cell lines using the cell filter device. The tumor cell suspensions and fluorescent labelled tumor cells mixed with blood or bone marrow suspensions were prepared and treated as described in the model experiments 1. and 2., and were subjected to the cell filter device. After washing, fixing and staining the cells on the filter in the device the filter was viewed by microscopy. The results from the tumor cell suspension alone showed antibody-bead-tumor cell complexes clearly isolated on the filter. The results from the fluorescent labelled tumor cell suspension together with blood or bone marrow also showed antibody-bead-tumor cell complexes clearly isolated on the filter (FIG. 6). Additional experiments to test the sensitivity of the method showed that 100 tumor cells, when mixed with a suspension of 10^7 blood or bone marrow cells, could be detected using this method.
4. Growth of separated cells isolated using the cell filter device. Tumor cell suspensions treated and isolated as described in the model experiments 1. and 2., were subjected to the filter device and the filter was subsequently incubated in a semi-solid medium containing 0.3% agarose in culture medium containing 20% calf serum. The cells were incubated in an atmosphere of 5% CO_2 at 37C. The tumor cells showed an ability to divide and grow.

In several experiments some unspecific binding to the mononuclear cells was observed, which was found to be unrelated to the nature of the specific antibody, and which was equally pronounced with SAM-coated particles alone. The magnitude of this unspecific binding varied from almost 0% to a level between 0.5–2%. This unspecific binding was almost eliminated by mild treatment with detergent, (TWEEN 20™ polyoxyethylenesorbitan monolaurate) performed to reduce the problem of hydrophobic cell interactions.

Examples of the Usefulness of the precedure

1. Detection of micrometastic neoplastic disease in blood and marrow. Early and reliable diagnosis of spread of cancer cells to blood and/or bone marrow has become increasingly important for the choice of optimal therapy, possibly curative in many types of cancer, including carcinomas, as described in application Example 1. Similar procedures for malignant melanoma, sarcoma, neuroblastoma and several other cancers have been established or are under development.
2. Detection of malignant cells in pleural or ascitic effusions and urine. The nature of such effusions may represent an important diagnostic problem, particularly when a low number of cancer cells are present together with normal reactive or epithelial cells. In several cases a definite diagnosis has been rapidly made with the new method, in cases where conventional cytological examination has been negative or inconclusive. A similar advantage can be found in cases of cancer in the kidneys or in the urinary tract and bladder.
3. Detection of neoplastic cells in the cerebrospinal fluid. As the systemic treatment of many cancer types have improved, the frequency of cases with symptom-giving brain metastases have significantly increased, and in parallell with this, the necessity for early detection of such spread. With the use of the new procedure even a low number of malignant cells can easily be identified, permitting intervention with therapeutic alternatives at an early stage of intracranial tumor manifestations.
4. Diagnosis of cancer in biopsied tissue. When cancer is suspected, and tissue biopsies are obtained by surgical procedures or by e.g. needle biopsies, a much more simple and rapid diagnosis can be made with the new method, used on prepared cell suspensions, compared to conventional morphological or immunohistochemical or cytochemical procedures. Distinction between several alternative cancers can be made by the use of the appropriate antibodies.
5. Identification of prognostic indicators. Since the expression of several membrane molecules have been shown to correlate with progression of the malignant disease in several cancers, the present method can be used to identify prognostic indicators, for example as described in application Example 2.
6. Identification of cells indicative of specific diseases or of disease progression or state. In various types of rheumatoid diseases (such as rheumatoid arthritis), as well as in allergic, autoimmune, and cardiovascular diseases, identification of the systemic or local presence of specific subpopulations of cells is important for diagnosis and for determining the stage of the disease. Rapid detection of such cell populations with the new method is therefore of considerable diagnostic and therapeutic importance.
7. Detection of subpopulations of normal cells. For several purposes, it will be important to detect the fraction of a particular subpopulation of normal cells in a population. This applies e.g. to liver biopsies where the identification of cells expressing the biliar epithelial antigen, may be of importance. Similarly, the identification, and possible isolation of specific endothelial cells from a cell suspension prepared from various normal tissues may be warranted.
8. Isolation and growth of selected cells. For many of the above mentioned purposes it may be required to have a

larger population of cells to study. The present method using the cell filter device can provide the conditions to permit the propagation of the positively selected target cells, without the presence of free unbound particles or other unspecifically bound cells.

Several of the cell membrane molecules mentioned above in sections 1–6 may also be used as targets for immunotherapy with several types of activated killer cells or for example with immunotoxins. The identification with the new method of expression of such molecules is, therefore, also of value for determining in which cases such types of therapy should be used.

Examples of A Practical Application of the Method

Example 1

To diagnose spread of cancer cells in blood and/or bone marrow at an early stage, we have used in the new procedure the MOC-31, NrLu10, BM2, BM7, 12H12, and MLuCl anti-carcinoma antibodies to determine whether or not micrometastatic disease from breast, lung, colorectal, and prostate cancer might be sensitively identified in such body fluids. The successful results with these antibodies have significant clinical implications.

Example 2

The expression of many cell membrane molecules have been shown to correlate with progression of the malignant disease in several types of cancer. The detection of binding of such molecules to respective antibodies can therefore be used to obtain information of high prognostic value. Among such antigens are a high number of adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors and carcinoma markers listed below. We have, with the new procedure identified the binding of particle-antibody complexes to CD44 variants, E-cadherin, Le^y, CEA, EGF-r. transferrin receptor, MUC-1 epitope, LUBCRU-G7 epitope, prostate cancer antigen, UJ13A epitope, 2-microglobulin, HLA-antigens, and apoptosis receptor.

Example 3

Two litres of pleural effusion from a patient supposed to suffer from malignant melanoma was obtained. After centrifugation, the cells were suspended in a volume of 2 ml of RPMI with a 10% fetal calf serum, incubated with 9.2.27 anti-melanoma antibody (10 g/ml) at 4C. for 30 min, washed and again incubated with Dynabeads™ SAM M450/IgG2A at 4C. for 30 min. The cell suspension was then examined under a microscope for determining the fraction of cells with paramagnetic cells attached to their surface. The diagnosis of malignant melanoma was confirmed, as about 10% of the cells had a significant number of bound particle-rosettes.

Example 4

Biopsied tissue was obtained from a subcutaneous tumor in a case with clinical indications of either small cell lung cancer or a malignant melanoma. A single cell suspension was prepared from the biopsy, divided in 2 fractions, one incubated with the 9.2.27 anti-melanoma antibody, and the other with MOC-31 anti-carcinoma antibody (both at 10 g/ml). The incubation was similar to that used in the example above. None of the cells incubated with the melanoma antibody bound any beads, whereas all tumor cells incubated with MOC-31 were positive.

Example 5

Biopsied tissue from a patient suspected to have malignant i5 melanoma was examined by preparing single cell suspension, incubating with 9.2.27 anti-melanoma antibody, and then following the procedure as above. Most of the cells were positive with a high number of particle-rosettes attached to their membranes

Example 6

A pleural effusion from a breast cancer patient was studied to examine whether tumor cells could be detected in the fluid. One litre of the fluid was centrifuged, the cells resuspended, and in separate vials incubate with each of 3 different anticarcinoma antibodies (MOC-31, 2E11, 12H12). After completing the procedure as in the previous example, it was found that most of the cells bound to antibody-coated particles in all 3 cases.

Example 7

A bone marrow suspension obtained from a breast cancer patient was studied to examine whether micrometastic tumor cells could be present. After the preparation of mononuclear cells, these were incubated with the same 3 anti-carcinoma antibodies used in the example above, but in this case the antibodies were first attached to Dynabeads™ SAM IgG paramagneteic particles. After 1 incubation with these directly coated particles, the cell suspension was examined in the microscope, and a high number of cells were found positive with a number of particlerosettes attached to their membrane. Similar experiments have been performed in a number of pleural or ascitic effusion and bone marrow from patients with breast.

Example 8

T47D human breast carcinoma cells were incubated for varying lenghts of time with Hoechst fluoresence dye, -and the viability of the labeled cells was checked. Varying numbers of labeled breast carcinoma cells were then added to 1×10^6 bone marrow cells obtained from healthy volunteers. In different experiments, different concentrations of paramagnetic, monodisperse particles (Dynabeads™ P450) coated with individual anticarcinoma antibodies (NrLulO, MOC31, or 12H12) were added. After incubation for 30 min on ice, samples of the different test tubes were examined in a counting chamber under light and fluorescence microscopy. When the ratio of tumor cells/total nucleated cells was low, the cell suspension was subjected to a magnetic field and the cells with particles attached were isolated before examined in the microscope. It was found that at an optimal ratio of 1–10 paramagnetic beads per tumor cell in the cell mixture, all the tumor cells had from 2–15 beads attached to their surface. The sensitivity of the detection method was close to one target-cell per 10^4 nucleated cells. In control experiments with labeled tumor cells using antibodies known to have some cross-reativity to normal cells, this cross-reactivity was confirmed with the antibody-coated paramagnetic particles. In experiments with beads without tumor-associated antibody coating, none of the target cells bound any beads.

Similar experiments have been performed both with other breast cancer lines and a small cell lung cancer cell line. Similar sensitivity and specificity were obtained in these experiments.

Example 9

Pleural and ascites fluid from patients with breast cancer and ovarian carcinoma were centrifuged, the same coated paramagnetic particles used in Example 1 were added,

incubated and concentrated in a magnetic field before the suspension was examined under light microscopy. Typically, cells that had the clear morphological features of tumor cells had beads attached, whereas none of the few normal cells bound the antibody-coated beads. In two cases with pleural effusion, an independent morphological examination did not reveal the presence of any tumor cells, whereas a significant number malignant cells were detected by the use of antibody-coated beads. In some cases, tumor cells were separated in a magnetic field and transferred to tissue culture flasks containing growth medium specially prepared for growing breast cancer cells, in attempts to establish permanent cell lines from these cultures. In parallel, cells from the malignant effuisions were cultivated directly without positive selection with magnetic beads. In the latter cases, no cell line could be established, whereas in more than 50% of the cases where positively selected tumor cells had been used, cell lines were successfully established.

Example 10

In some cases, bone marrow and peripheral blood obtained from patients with breast cancer were examined with the present procedure by adding antibody-coated paramagnetic beads, incubating for 30 min at 4C. and concentrating in a magnetic field and by examining the suspension under light microscopy. In both cases binding of the paramagnetic beads to tumor cells, representing 0.1–1% of the nucleated cells in the bone marrow and blood was detected, cells that could not be identified by any other method.

Example 11

Antibodies against certain growth factor receptors or other gene products expressed on the surface of specific cell populations may be used to identify and positively select these cells. Beads coated with anti-transferrin receptor antibodies, used in the novel method according to the present invention were shown to represent a rapid, simple and sensitive method for identification of cells expressing the transferrin receptor.

Example 12

For various purposes isolation of specific populations of normal cells is warranted. Endothelial cells lining the capillary or small vessels in normal or tumorous tissue could be positively selected from cell suspensions prepared from the relevant tissues. The procedure involved the use of beads coated with antibody directed against structures expressed on the endothelial cells, but not on the other normal cells in the cell mixture.

Example 13

Human cells injected into immunodeficient rodents was shown to be present in cell suspensions prepared from tumor xenografts and from various host organs/tissues by employing magnetic particles coated with an anti-pan human antibody.

Example 14

Tumor cell lines from breast carcinoma and melanoma patients were separated from a mixed population of blood or bone marrow cells and filtered using the cell filter device described. After the addition of culture medium and subsequent incubation the selected tumor cells on the filter were able to grow in the absence of free unbound particles or other unspecifically bound cells.

TABLE 1

List of relevant antigens and examples of associated antigen-binding antibodies

ANTIGENS	MONOCLONAL ANTIBODIES
Adhesion molecules	
Fibronectin receptor (=5β1 integrin)	Pierce 36114, BTC21/22
	Calbiochem 341649
Integrin α3β1	M-Kiol 2
Vitronectin receptor (αvβ3 integrin)	TP36.1, BTC 41/42
Integrin α2	Calbiochem 407277
Integrin α3	Calbiochem 407278
Integrin α4	Calbiochem 407279
Integrin α5	Calbiochem 407280
Integrin αV	Calbiochem 407281
Integrin β2	Calbiochem 407283
Integrin β4	Calbiochem 407284
GpIIβIIIα	8221
ICAM-I (CD54)	C57–60, CL203.4, RR 1/1[1]
VCAM-1	Genzyme 2137-01
ELAM-1	Genzyme 2138-01
E-selectin	BBA 8
P-selectin/GMP-140	BTC 71/72
LFA-3 (CD58)	TS 2/9
CD44	BM 1441 272, 25:32
CD44-variants	11.24, 11.31, 11.10
N-CAM (CD56)	MOC-1
H-CAM	BCA9
L-CAM	BM 1441 892
N-CAM	TURA-27
MACAM-1	NKI-M9
E-cadherin	BTC 111, HECD-1, 6F9
P-cadherin	NCC-CAD-299
Tenascin	BM 1452 193
	Calbiochem 580664
Thrombospondin receptor (CD36)	BM 1441 264
VLA-2	A1.43
Laminin receptor	
HNK-1 epitope	HNK-1
Carbohydrate antigens	
T-antigen	HH8, HT-8
Tn-antigen	TKH6, BaGs2
Sialyl Tn	TKH-2
Gastrointestinal cancer associated antigen (M_w200kD)	CA 19-9
Carcinoma associated antigen	C-50
Le^y	MLuC1, BR96, BR64
di-Le^x, tri-Le^x	B3
Dimetric Le^a epitope	NCC-ST-421
H-type 2	B1
CA15-3 epitope	CA15-3
CEA	I-9, I-14, I-27, II-10, I-46
	Calbiochem 250729
Galbl-4GlcNac (nL4,6,8)	1B2
H-II	BE2
A type 3	HH8
Lacto-N-fucopentanose III (CD15)	PM-81
Glycolipids	
GD_3	ME36.1, R24
GD_2	ME36.1, 3F8, 14.18
Gb_3	38-13
GM_3	M2590
GM_2	MKI-8, MKI-16
$FucGM_1$	1D7, F12
Growth factor receptors	
EGF receptor	425.3.2.E9, 225
c-erbB-2 (HER2)	BM 1378 988, 800 E6
PDGFα receptor	Genzyme 1264-00
PDGFβ receptor	Sigma P 7679
Transferrin receptor	OKT 9, D65.30
NGF receptor	BM 1198 637
IL-2 receptor (CD25)	BM 1295 802, BM 1361 937
c-kit	BM 428 616, 14 A3, 1D9.3D6
TNF-receptor	Genzyme 1995-01, PAL-M1

TABLE 1-continued

List of relevant antigens and examples of associated antigen-binding antibodies

ANTIGENS	MONOCLONAL ANTIBODIES
NGF-receptor	
Melanoma antigens	
High molelcular weight antigen (HMW 250.000)	9.2.27, NrML5, 225.28 763.74,. TP41,2, IND1
Mw105 melanoma-associated glycoprotein	ME20
100kDa antigen (melanoma/carcinoma)	376.96
gp 113	MUC 18
p95–100	PAL-M2
Sp75	15.75
gr 100–107	NKI-bereb
MAA	K9.2
M_r125kD (gp125)	Mab 436
Sarcoma antigens	
TP-1 and TP-3 epitope	TP-1, TP-3
M_w200kD	29–13, 29.2
M_w160kD	35–16, 30–40
Carcinoma markers	
MOC-31 epitope (cluster 2 epithelial antigen)	MOC-31, NrLu10
MUC-1 antigens (such as DF3-epitope (gp290kD)	MUC-1, DF3.BCP-7 to -10
MUC-2 and MUC-3	PMH1
LUBCRU-G7 epitope (gp 230kD)	LUBCRU-G7
Prostate specific antigen	BM1276 972
Prostate cancer antigen	E4-SF
Prostate high molecular antigen M_w > 400kD	PD41
Polymorphic epithelial mucins	BM-2, BM-7, 12-H-12
Prostate specific membrane antigen (Cyt-356)	7E11-C5
Human milk fat globulin	Immunotech HMFG-1, 27.1
42kD breast carcinoma epitope	B/9189
$M_{w>10^6}$ mucin	TAG-72, CC-49, CC-83
Ovarian carcinoma OC125 epitope (M_w750 kD)	OC125
Pancreatic HMW glycoprotein	DU-PAN-2
Colon antigen Co17-1A(M_w37000)	17-1A
G9-epitope (coloncarcinoma)	G9
Human colonic sulfomucin	91.9H
M_r300kD pancreas antigen	MUSE11
GA 733.2	GA733,KS1.4
TAG 72	B72.3, CC49, CC83
Undefined	Oa11, SM1
Pancreatic cancer-associated	MUSE 11
Pancarcinoma	CC49
Prostate adenocarcinoma-antigen	PD 41
M_w150–130kD adenocarcinoma of the lung	AF-10
gp 160 lung cancer antigen (Cancer Res. 48, 2768, 1988)	anti gp160
M_w92kD bladder carcinoma antigen	3G2-C6
M_w600kD bladder carcinoma antigen	C3
Bladder carcinoma antigen (Cancer Res. 49, 6720, 1989)	AN43, BB369
CAR-3 epitope M_w > 400kD	AR-3
MAM-6 epitope (C15.3)	115D8
High molecular ovarian cancer antigen	OVX1, OVX2
Mucin epitope Ia3	Ia3
Hepatocellular carcinoma antigen M_w900kD	KM-2

TABLE 1-continued

List of relevant antigens and examples of associated antigen-binding antibodies

ANTIGENS	MONOCLONAL ANTIBODIES
Hepemal epitope (gp43) Hepato-cellular carc. ag	Hepema-1
O-linked mucin containing N-glycolylneuraminic acid	3E1.2
M_w48kD colorectal carcinoma antigen	D612
M_w71kD breast carcinoma antigen	BCA 227
16.88 epitope (colorectal carcinoma-antigen)	16.88
CAK1 (ovarian cancers)	K1
Colon specific antigen p	Mu-1, Mu-2
Lung carcinoma antigen M_w350–420kD	DF-L1, DF-L2
gp54 bladder carcinoma antigen	T16
gp85 bladder carcinoma antigen	T43
gp25 bladder carcinoma antigen	T138
Neuroblastorna antigens	
Neuroblastoma-associated, such as UJ13A epitope	UJ13A
Glioma antigens	
Mel-14 epitope	Mel-14
Head and neck cancer antigens	
M_w18–22kD antigen	E48
HLA-antigens	
HLA Class 1	TP25.99
HLA-A	VF19LL67
HLA-B	H2-149.1
HLA-A2	KS1
HLA-ABC	W6.32
HLA-DR, DQ, DP	Q 5/13, B 8.11.2
β2-microglobulin	NAMB-1
Apoptosis receptor	
Apo-1 epitope	Apo 1
Various	
Plasminogen activator antigens and receptors	Rabbit polylonal
p-glycoprotein	C219, MRK16JSB-1, 265/F4
cathepsin D	CIS-Diagnostici, Italy
biliary epithelial antigen	HEA 125
neuroglandular antigen (CD63)	ME491, NKI-C3, LS62
CD9	TAPA-1, R2, SM23
pan-human cell antigen	pan-H